Role of BAALC Gene in Prognosis of Acute Lymphoblastic Leukemia in Egyptian Children

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Abstract Acute lymphoblastic leukemia (ALL) is characterized by excess bone marrow lymphoblast cell production. Brain and Acute Leukemia Cytoplasmic gene (BAALC gene) is a recently identified gene on chromosome 8q22.3. The aim of this work was to study the role of BAALC gene in prognosis of ALL in Egyptian children. This study was conducted on 60 children with ALL who were admitted in Oncology Unit in the period from January 2012 to March 2015 including 36 males and 24 females with mean age of 8.74 ± 2.66 years. For all patients the following were done: Full history taking, thorough clinical examination and laboratory investigations including: complete blood count, LDH, bone marrow aspiration, cytochemistry, immunophenotyping, FISH technique for detection of t(12;21) and t(9;22) and assessment of BAALC gene expression by PCR in bone marrow aspirate mononuclear cells at diagnosis before chemotherapy. Positive BAALC gene expression was found in 36 patients (60%) and negative expression in 24 patients (40%). Positive BAALC gene expression group includes 14 males and 22 females with mean age at presentation of 8.45 ± 2.77 years while negative BAALC gene expression group includes 18 males and 6 females with mean age at presentation of 8.61 ± 2.44 years with no significant differences between positive and negative BAALC gene expression groups regarding age, sex, clinical presentations, WBCs and platelets counts, hemoglobin and

LDH levels, peripheral blood and BM blast cell counts, immunophenotyping and chromosomal translocations including t(12;21) and t(9;22). There were significant differences in disease outcome between positive and negative BAALC gene expression groups with higher rate of relapse and death and lower rate of complete remission, disease free survival (DFS) and overall survival (OS) in positive BAALC gene expression group compared with negative group ($p = 0.028$). Multivariate analyses for overall survival and disease free survival shows significant role of BAALC gene expression in OS and DFS. BAALC expression might represent an additional prognostic marker in children with ALL and should be routinely assessed at diagnosis for better prognostic assessment.

Keywords Acute lymphoblastic leukemia (ALL) · BAALC gene expression · Prognostic factors of ALL

Introduction

ALL is the most common cancer diagnosed in children and represents approximately 25% of cancer diagnoses among children younger than 15 years [1]. With improvements in diagnosis and treatment, overall cure rates for children with ALL have reached 90% [2]. The use of risk-adapted treatment protocols has improved cure rates while limiting the toxicity of therapy.

Brain and acute leukemia cytoplasmic gene (BAALC) which maps on chromosome 8 at 8q22.3 was originally observed in the neuroderm and its expression was reported in early hematopoietic cells of CD 34+ (cluster of differentiation) however, no expression was observed in the peripheral mononuclear cells [3].

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An elevated expression of BAALC gene was originally discovered in a gene expression profiling study of AML with trisomy 8 [4]. Overexpression of BAALC gene was also seen in other types of AML and ALL suggesting a role for BAALC gene overexpression in acute leukemia [5]. BAALC gene over expression predicts an inferior outcome in AML and ALL [6–8].

Additionally, high BAALC gene expression occurs in glioblastoma, melanoma, and childhood gastrointestinal stroma tumors, suggesting an oncogenic role for BAALC gene [9].

The function of BAALC gene in hematopoietic system, its contribution in leukemogenesis and the pathogenic impact of high BAALC gene expression and its association with different adverse prognosticators in the evolution of leukemia remains elusive, since neither the role of BAALC gene nor the role of most of the concomitant mutations in leukemogenesis have been fully clarified. Recently, it was proposed that BAALC gene blocks myeloid differentiation but does not promote cell proliferation and thus requires a second mutation, which gives a proliferative advantage to induce leukemia [4, 10]. Subsequently researches have shown that the highly expressed BAALC gene is associated with minimal residual disease, which leads to a low complete remission rate and overall survival [3].

As prognostic value of BAALC gene expression was extensively studied in AML with inferior outcome in case of BAALC gene expression [5] but no sufficient studies were done in ALL especially in pediatric ALL. Therefore, our aim in the present study was to evaluate the prognostic impact of BAALC gene expression on clinical outcome of childhood ALL in Egyptian children.

Patients and Methods

Approval for this study was granted by Ethical Committee of Tanta University research center and written consent was obtained from the parents of all children involved in this study. The study participants included 60 Egyptian children being followed up under the Oncology Unit of the Pediatric Department at Tanta University including 36 males and 24 females with an age range of 4–15 years and mean age value of 8.74 ± 2.66 years, in the period from January 2012 to March 2015. Criteria for inclusion into the cohort were diagnosis of acute lymphoblastic leukemia. All study participants were subjected to thorough clinical examination, paying particular attention to fever, pallor, purpura, hepatomegaly, splenomegaly, and lymphadenopathy. Study participants were also subjected to laboratory investigations including complete blood count, Lactate dehydrogenase (LDH), bone marrow aspiration with cytochemical examination, immunophenotyping,

Fluorescent In Situ Hybridization (FISH) for detection of t(9;22) and t(12;21) and real time polymerase chain reaction (RT-PCR) for assessment of BAALC Gene.

Specimen Collection and Handling

Three ml venous bloods were collected under complete aseptic technique. They were delivered into 2 tubes: 1 ml blood into tube containing EDTA for complete blood count and 2 ml blood into plain tube for assessment of LDH. Two ml of bone marrow aspirate were drawn into a sterile tube containing EDTA for mononuclear cell separation for PCR.

Immunophenotyping

Immunophenotyping was performed on gated blast cells from bone marrow samples by flow cytometry using an extensive panel of Fluorescein Isothiocyanate (FITC) and Phycoerythrin (PE) conjugated monoclonal antibodies including T cell lymphoid markers (CD2, CD3, CD5, CD7, CD4, CD8), B-cell markers (CD10, CD19, CD22 and cyto immunoglobulin) and myeloid cell markers (CD13, CD33, cyto MPO). All MoAbs were purchased from (BD Science, San Jose, CA) [11].

Fluorescent In Situ Hybridization

BM specimens were cultured for 72 h at 37 °C in Roswell Park Memorial Institute medium (RPMI medium) supplemented with 10% fetal bovine serum without addition of any mitogen (un-stimulated). Colcemid (0.02 µg/ml) was added to the cultures 30 min before harvest. After 30 min of hypotonic treatment with 0.075 M KCL, the cells were fixed with methanol and acetic acid (3:1) and cells were made into slide preparations. Hybridization mixture (10 µl) was then applied to each slide, which was covering slipped and sealed. Hybridization solution contained hybridization buffer, purified water, and the specific probe. Specific LSI BCR-ABL dual color DNA probe hybridizes to chromosome 9q³⁴ and chromosome 22q^{11.2} to detect t(9;22)(q³⁴,q^{11.2}) and TEL-AML dual color DNA probe hybridizes to chromosome 12p¹³ and chromosome 21q²² to detect t(12;21)(p¹³,q²²) were used. FISH assay was performed according to (Vysis, Abbott # 32-190022) manufacturer's instructions. Analysis of cells was done under fluorescence microscope equipped with Quips spectra vision hardware and software [12] (Fig. 1).

Assessment of BAALC Gene

Mononuclear cells were separated from the samples by centrifugation on Density gradient medium [13]. RNA was isolated using an RNA easy Mini Kit and the concentration

Fig. 1 FISH technique showing a case of TEL/AML fusion genes (left part of the figure) and a case of BCR/ABL fusion genes (right part of the figure)

of extracted RNA was evaluated by spectrophotometry (SPEC) [14]. DNA amplification was done by real time PCR using Gene Amp 5700 Sequence Detection System. Real time PCR was used for the detection of BAALC gene [15].

BAALC mRNA expression was normalized to the simultaneously analyzed glucose phosphate isomerase (GPI gene). The relative BAALC expression was determined using the comparative cycle threshold method. BAALC were amplified using 1 μ l cDNA, 1 $\times$ master mix (IQ Mix; BioRad, Munich, Germany).

Glucose phosphate isomerase (GPI) forward primer:

5'-TCTTCGATGCCAACAAGGAC-3';

Glucose phosphate isomerase (GPI) reverse primer:

5'-GCATCACGTCTCCGTCAC-3';

Glucose phosphate isomerase (GPI) probe:

5'HEX-TTCAGCTTGACCCTCAACACCAAC-TAMRA-3';

BAALC Gene forward primer:

5'-GCCCTCTGACCCAAACAG-3';

BAALC Gene reverse primer:

5'-CTTTTGCAGGCATTCTCTTAGCA-3';

BALCC Gene probe:

5'-FAMCTCTTTAGCCTCTGTGGT-3';

Reactions were performed using real-time PCR 7000 sequence detection system (Applied Biosystems, Foster city, CA, USA) [11]. The expression BAALC gene is calculated relative to Housekeeping gene, and so the value as a ratio to this gene is either expressed (positive BAALC gene expression) or not expressed (negative BAALC gene expression). BAALC gene expression in peripheral blood was examined from 10 healthy children as control, and the cut-off value is determined relative to controls. BAALC gene value of 0.166 was considered as cut off value so high or positive BAALC gene expression was considered if

BAALC gene expression is above 0.166, whereas low or negative BAALC gene expression was considered if BAALC gene expression is below 0.166. The blue line in Fig. 2 denoting the threshold cycle at which there was significant increase in the fluorescence which is 0.050225 (Fig. 2).

Follow up of patients was carried out clinically and by blast cell count in BM on day 21 of induction therapy which includes Vincristine 1.5 mg/kg/m²/week IV (days 0, 7, 14, 21, 28, 35), Doxorubicin 25 mg/m²/week IV infusion (days 0, 7, 14, 21, 28, 35), asparaginase 6000 u/m² SC on alternate days (10 doses) and oral prednisone 40 mg/m²/day for 6 weeks. If no complete remission on day 21 and BM contains more than 5% blast cells, we add etoposide 100 mg/m²/dose IV (days 22, 25, 29), cyclophosphamide 750 mg/m²/dose IV infusion (days 22, 25, 29), aracytin 100/m²/dose IV (days 22, 25, 29), and methotrexate 5 g/m² over 4 h on day 28 [14]. Follow up was continued during the whole period of chemotherapy which includes 9 weeks consolidation [16, 17], 6 weeks interim maintenance [17], 6 weeks delayed intensification [18] and 30 months maintenance therapy [19] to detect relapse [18]. In patients with t(9; 22); Imatinib (Gleevec) was given in combination with chemotherapy in dose of 340 mg/m²/day (not exceed 600 mg) till complete remission [20] followed by allogeneic hematopoietic stem cell transplantation after first remission in patients with t(9;22) if suitable matched donor is available [21].

Statistical Analysis

The patient's data were collected and statistically analyzed using SPSS software version 12. All Data were expressed as in terms of mean values $\pm$ SD. The difference between two means was statistically analyzed using the student (*t*) test. Chi square test (X^2) and Fischer exact test was used

Fig. 2 Positive expression of BAALC by quantitative real-time PCR. Amplification Plot showing the log of the change in the fluorescence plotted versus cycle number (ΔRn vs. Cycle)

as a test of significance. Log rank test were used to assess survival. Significance was adopted at $p \leq 0.05$.

Results

There were significant differences between patients with positive and negative BAALC gene expression regarding age and sex with older age in positive BAALC gene group and more BAALC gene expression in females but no significant differences regarding clinical presentations at diagnosis (Table 1).

There were no significant differences between positive and negative BAALC gene expression groups regarding platelets counts, hemoglobin and LDH levels, but positive BAALC gene expression group has significantly higher WBCs and peripheral blood and BM blast cell counts at diagnosis and BM blast cells after induction therapy compared with negative BAALC gene expression group (Table 2).

There was no significant association between positive BAALC gene expression and immunophenotyping and chromosomal translocations (Table 2).

Positive BAALC gene expression group has significantly higher relapse and death rates and lower rate of complete remission, overall survival and disease free survival compared with negative group ($p = 0.028$) (Table 3; Fig. 3).

Multivariate analyses for overall survival and disease free survival in studied patients with ALL shows significant role of BAALC gene expression in OS and DFS in patients with ALL (Table 4).

Discussion

ALL is the most common childhood malignancy. The outcome of treatment for ALL in children is generally favorable, with cure rates exceeding 80%. Consequently, there is great interest in identifying clinical, laboratory and genetic markers that may distinguish patients likely to be cured, who require less intensive treatment, from patients at high risk of relapse, who require aggressive or novel therapies [22].

The present study was designed to study the role of BAALC gene expression in prognosis of ALL in Egyptian children.

Table 1 Comparison between patients with positive and negative BAALC gene expression regarding clinical features

	Positive BAALC (n = 36)	Negative BAALC (n = 24)	<i>t</i> test or χ^2	<i>p</i> value
Age (mean $\pm$ SD)	8.45 $\pm$ 2.77	6.22 $\pm$ 2.44	0.435	0.049*
Sex				
Males	14 (23.33%)	18 (30%)	0.270	0.031* ^a
Females	22 (36.66%)	6 (10%)		
Pallor	29 (80.55%)	19 (79.16%)	0.30	0.554
Purpura	31 (86.11%)	21 (87.5%)	3.44	0.131
Lymphadenopathy	28 (77.77%)	17 (70.83%)	0.266	0.477
Hepatomegaly	27 (75%)	17 (70.83%)	0.088	0.643
Splenomegaly	29 (80.55%)	20 (83.33%)	0.48	0.468
Bone-ache	12 (33.33%)	7 (29.16%)	0.77	0.538

SD, standard deviation; Positive BAALC, positive BAALC gene expression; Negative BAALC, negative BAALC gene expression

^a Between males and females in the same group

This study revealed significant difference between BAALC gene expression regarding sex with more BAALC gene expression in females. This is similar to Ben Abdelali et al. [23] and Baldus et al. [7] who found that females are more frequently express BAALC gene than males.

In this current study, older children presented at diagnosis more frequently a positive BAALC gene expression. Kühnl et al. [24] found the same results while Baldus et al. [7] found no significant difference in pretreatment age between low and high BAALC expression.

No significant differences were found between positive and negative BAALC gene expression groups regarding clinical presentations at time of diagnosis. No enough references in BAALC gene expression in ALL for comparison but Yahya et al. [25] and Hagag et al. [26] found no significant differences between positive and negative BAALC gene expression groups regarding clinical presentation in patients with AML.

No significant differences were found between positive and negative BAALC gene expression groups regarding platelets counts, hemoglobin and LDH levels, but there was significantly higher WBCs and blast cell counts in the peripheral blood and BM at time of diagnosis and BM blasts after induction in positive BAALC gene expression group; this was in agreement with Kühnl et al. [24] who found significantly higher initial WBCs count in patients with high BAALC expression and Baldus et al. [7] who found significantly higher initial peripheral blood blast cells in high BAALC gene expression group.

Out of studied 60 patients with ALL; 49 (81.6%) were found to be of B cell origin and 11 (18.33%) of T-cell origin with no significant association between BAALC gene expression and immunophenotyping. Higher prevalence of B cell ALL in our study is similar to Ratei et al. [27] who found B-cell precursor ALL in 82.6% and T-cell ALL in 14.7% and Donald and Mahoney [28] who found

T-cell ALL in 15%. Of Limited number of studies that was done regarding the association between BAALC gene expression and immunophenotyping, Baldus et al. [7] reported high BAALC gene expression in early T-ALL.

Significantly lower CR and significantly higher relapse and death rates were found in positive BAALC gene expression which could be explained by the association between BAALC gene expression and older age and higher WBCs count at time of diagnosis which is well known bad prognostic variables. Baldus et al. [7] and Kühnl et al. [24] found significantly lower CR rate and shorter overall survival and significantly higher incidence of primary resistant disease and higher relapse rate in patients with ALL with high BAALC gene expression and Santamaría et al. [29] found lower CR rates, poor overall survival and disease free survival in patients with AML with high BAALC gene expression [27] which could be explained by the association of high BAALC gene expression at time of diagnosis with minimal residual disease after the induction therapy [3] while Ben Abdelali et al. [23] found no significant influence of BAALC expression upon prognosis in their study that included 232 adults with T cell ALL.

The differences in the results between this work and previous studies may be due to different behavior and clinical presentation of leukemia in different localities, different number of studied patients and different time of research in different studies.

Limitation of the Study

Number of patients included in this study is limited and need to be more and more.

The period of follow up to predict the prognosis (2 years) is short and need to be longer for better assessment of the role of BAALC gene in prognosis of ALL.

Table 2 Comparison between patients with positive and negative BAALC gene expression regarding some laboratory data

	Positive (n = 36)	Negative (n = 24)	<i>t</i> test or χ^2	<i>p</i> value
BAALC gene expression				
Range	0.167–3.22	0.02–0.165		
Mean $\pm$ SD	2.023 $\pm$ 0.34	0.049 $\pm$ 0.026	3.24	0.022*
Median	1.82	0.060		
Hemoglobin				
Range	5–11	6–12		
Mean $\pm$ SD	9.44 $\pm$ 1.24	8.99 $\pm$ 1.26	4.22	0.91
Platelets ($\times 10^3/\text{mm}^3$)				
Range	30–120	45–132		
Mean $\pm$ SD	59.86 $\pm$ 33.12	61.13 $\pm$ 48.26	1.77	0.871
TLC ($\times 10^3/\text{mm}^3$)				
Range	23–172	17–103		
Mean $\pm$ SD	85.34 $\pm$ 41.22	63.22 $\pm$ 43.31	0.88	0.042*
Blast cells (%) in peripheral blood				
Range	2–80	0–67		
Mean $\pm$ SD	66.19 $\pm$ 12.21	33.15 $\pm$ 18.48	0.962	0.035*
BM blast cells (%) at diagnosis				
Range	44–92	37–80		
Mean $\pm$ SD	81.22 $\pm$ 7.99	69.49 $\pm$ 11.89	0.933	0.048*
BM blast cells (%) after induction				
Range	2–5	1–4		
Mean $\pm$ SD	3.94 $\pm$ 0.99	2.52 $\pm$ 0.67	3.61	0.049*
LDH (U/L)				
Range	980–1600	880–1240		
Mean $\pm$ SD	1214.51 $\pm$ 388.63	1189.57 $\pm$ 266.47	0.89	0.088
Immunophenotyping				
Early Pre B: 33 (55%)	20 (33.33%)	13 (21.66%)	2.34	0.29
Pre B cell: 16 (26.66%)	10 (16.66%)	6 (10%)	3.32	0.33
T cel: 11 (18.33%)	6 (10%)	5 (8.33%)	4.71	0.47
Chromosomal translocation				
t(12;21): 15 (25%)	9 (25%)	6 (25%)	3.4	1.00
t(9;22): 3 (5%)	2(5.55%)	1 (4.16%)	2.34	0.9

Positive, positive BAALC gene expression; Negative, negative BAALC gene expression

SD standard deviation

* Significant ($p < 0.05$)**Table 3** Outcome of studied patients in relation to BAALC gene expression

	Positive BAALC expression (n = 36) (60%)	Negative BAALC expression (n = 24) (40%)
Death (15 cases)	11 (18.33%)	4 (6.66%)
Remission (34 cases)	16 (26.66%)	18 (30%)
Relapse (11 cases)	9 (15%)	2 (3.33%)
Chi square		
χ^2	6.332	
<i>p</i> value	0.028*	

* Significant

Fig. 3 Kaplan Meir curve showing time of death and relapse within 24 months of follow up in BAALC gene expression groups

Time for death and relapse in the studied groups within 24 months of follow up

Table 4 Multivariate analyses for overall survival and disease free survival in studied patients with ALL

Co-parameters	Overall survival				Disease free survival			
	<i>p</i>	HR	95% CI		<i>p</i>	HR	95% CI	
Age	0.72	0.842	0.872	1.232	0.832	0.898	0.955	1.123
Initial BM Blasts (%)	0.93	1.210	0.965	1.124	0.154	1.211	0.976	1.030
Initial peripheral blasts (%)	0.84	2.863	1.211	3.754	0.287	1.765	0.895	3.212
Initial immunophenotyping								
Early pre B	0.083	1.341	0.896	1.87	0.25	1.211	0.879	1.77
Pre B	0.069	1.622	0.634	2.867	0.38	0.321	0.176	2.982
T cell	0.091	1.321	0.879	1.186	0.27	1.187	0.843	4.947
BAALC gene at diagnosis (positive versus negative)	0.032*	2.654	1.273	3.762	0.027*	1.122	1.129	3.268

* Significant

Cytogenetic study in this study included only t(12;21) and t(9;22) and it must be expanded to include other structural and numerical chromosomal abnormalities.

Conclusion and Recommendation

BAALC expression might represent an additional prognostic marker in children with ALL and should be routinely assessed at diagnosis for better prognostic assessment.

Compliance with Ethical Standards

Conflict of interest No conflict of interest has been declared by all authors.

Ethical Approval All procedure performed in the study involving human participants were in accordance with the ethical standards of ethical committee of research center in Tanta University and with the 1964 Helsinki declaration and its later amendments.

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